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Delta-like Protein 3 Prevalence in Small Cell Lung Cancer and DLL3 (SP347) Assay Characteristics

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• ***Context.***—**Delta-like protein 3 (DLL3) is a protein that is implicated in the Notch pathway.**

Objective.—**To present data on DLL3 prevalence in small cell lung cancer and staining characteristics of the VENTANA DLL3 (SP347) Assay. In addition, the assay's immunoreactivity with other neoplastic and nonneoplastic tissues is outlined.**

Design.—**Individual formalin-fixed, paraffin-embedded specimens of small cell lung cancer and tissue microarrays comprising neoplastic and nonneoplastic tissues were procured. Sections were cut and stained with DLL3 (SP347) assay. The slides were examined to determine prevalence, staining characteristics, and immunoreactivity.**

Results.—**Cytoplasmic and/or membranous staining was observed in 1040 of 1362 specimens of small cell lung cancer (76.4%). Homogenous and/or heterogeneous and partial and/or circumferential granular staining with varied intensities was noted. Immunoreactivity was also observed in other neoplastic and nonneoplastic tissues.**

Conclusions.—**Our study findings provided the profile of DLL3 staining characteristics that can be used for determining the level of DLL3 expression in small cell lung cancer.**

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In the United States, lung cancer is diagnosed in more than 222 500 patients and accounts for 155 870 deaths annually.[1] Small cell lung cancer (SCLC) is a distinct type of lung cancer that represents approximately 15% of all primary lung cancers.[2] It is a neuroendocrine tumor that is histologically defined by distinctive features including finely granular chromatin, nuclear molding, crush artifact, and Azzopardi phenomenon. Although treatment exists for primary SCLC, relapses are common; and despite the aggressive nature of SCLC, limited options are available as second-line treatment for this aggressive disease, with no third-line treatment available.[3–6] Because of the lack of treatment options for SCLC, it is imperative to further examine the biology of SCLC cancers and investigate new therapeutic targets for SCLC.

Delta-like protein 3 (DLL3) is a transmembrane protein that has been implicated in SCLC tumorigenesis through its interactions with the Notch pathway. Specifically, DLL3 inhibits Notch activation, thereby promoting neuroendocrine tumorigenesis.[7] This is in contrast to the other Notch family ligands (DLL1, DLL4, JAG1, and JAG2), which activate the Notch pathway in neuroendocrine tumors, leading to the suppression of tumor growth in these tumors.[8] DLL3's contribution to neuroendocrine tumorigenesis is further supported by studies demonstrating that it is a downstream target for achaete-scute homolog 1 (*ASCL1*), which has been linked with neuroendocrine cell fate decisions and tumorigenesis in SCLC.[9]

Studies have suggested that DLL3 is elevated in neuroendocrine tumors, including SCLC, whereas it is low in the majority of normal tissue in humans.[8] This presents an opportunity to target and selectively deliver drugs to tumor cells highly expressing the DLL3 antigen by using antibody-drug conjugates specific to the DLL3 antigen. Recently, we used a rabbit monoclonal antibody in the development of the VENTANA DLL3 (SP347) Assay, which may be used to identify patients with high levels of DLL3 expression in SCLC. Here, we present data on the prevalence of DLL3 expression in SCLC, as well as the staining characteristics and immunoreactivity of the DLL3 (SP347) assay.

MATERIALS AND METHODS

Specimen Cohort

Validation Cohort.—A validation cohort of 1503 cases of formalin-fixed (10% neutral buffered formalin), paraffin-embedded SCLC whole-section samples was procured from an internal tissue bank at Ventana Medical Systems, Inc, and the following commercial tissue banks: Asterand Bioscience; Avaden BioSciences; Boca Biolistics; Conversant Bio; GLAS; ILSbio; Impath; PrecisionMed, Inc; TriMetis Life Sciences; and US Biomax, Inc. The cohort included primary and metastatic SCLC samples and consisted of resections, core needle biopsies, and fine-needle aspirates. Review of hematoxylin-eosin–stained slides from cases in the validation cohort confirmed morphologic features consistent with small cell carcinoma. These include small to medium-sized cells with minimal cytoplasm, indistinct nucleoli, and nuclear molding.

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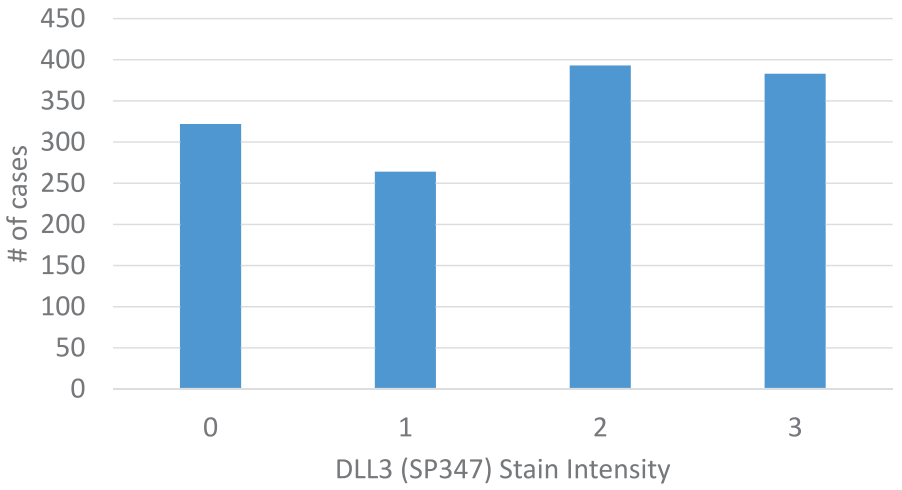

Figure 1. *Number of small cell lung cancer cases versus delta-like protein 3 (DLL3) (SP347) staining intensity (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining).*

Discovery Cohort.—The discovery cohort used for immunoreactivity assessments included the following: (1) Cut slides (4 μm) from 2 tissue microarrays (TMAs) of normal tissue: one array (FD808i-1) containing 24 types of normal human tissue representing 72 individual cases, and another array (999l, Food and Drug Administration) containing 32 types of normal human tissue representing 78 individual cases procured from US Biomax. (2) Cut slides (4 μm) also procured from a tumor tissue array (808i-2, Food and Drug Administration) containing 54 cases of carcinoma from multiple locations plus 18 cases with 6 types of normal tissue adjacent to tumor (single cores per case) representing 72 individual cases, also from US Biomax. (3) Cut slides (4 μm) from an array (LUC1504) of SCLC tissues containing 150 cores including 5 normal/benign lung tissue and 70 cases of SCLC tissue procured from Pantomics.

Immunohistochemistry Staining

Four-micrometer-thick tissue sections were cut from the cohort of cases and mounted on positively charged glass slides. The slides from both validation and discovery cohorts were stained with VENTANA DLL3 (SP347) Assay (P/N 790-7016, Ventana) along with OptiView DAB IHC Detection Kit (P/N 760-700, Ventana) on the BenchMark ULTRA automated staining platform using the recommended staining conditions of the DLL3 (SP347) assay package insert. Rabbit monoclonal negative control Ig (P/N 790-4795) was used as the negative reagent control. The samples were counterstained with hematoxylin II (P/N 790-2208, Ventana) and bluing reagent (P/N 760-2037, Ventana). Recommended tissue controls were used based on the DLL3 (SP347) assay package insert. For each case in the cohort and for the TMAs, one slide was stained with hematoxylin-eosin using either the VENTANA SYMPHONY system or Sakura Tissue-Tek Prisma & Film Automated Slide Stainer & Coverslipper according to the manufacturer's instruction.

Scoring

Validation Cohort.—Negative reagent control and DLL3 (SP347) assay–stained slides were evaluated for nonspecific background staining and cross-reactivity on a scale of 0 to 3 in increments of 0.5. Background staining was considered staining of

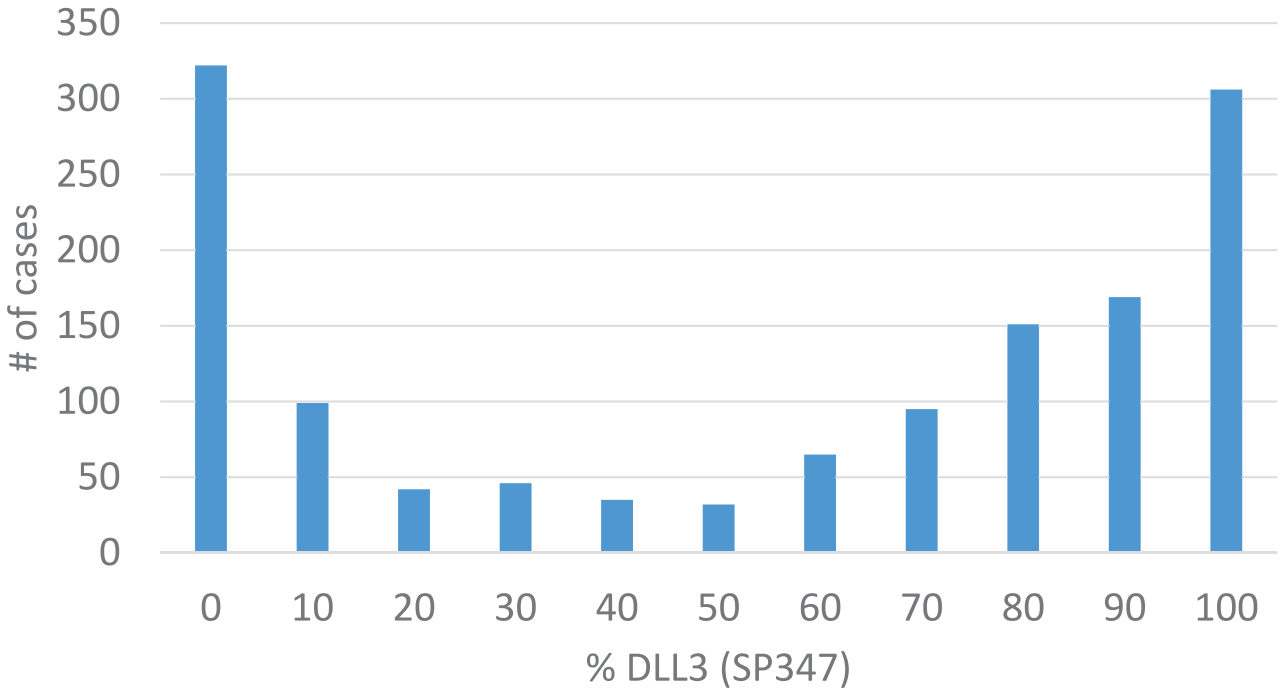

Figure 2. *Number of small cell lung cancer cases versus percentage tumor cell of delta-like protein 3 (DLL3) (SP347) staining at various intensities.*

Table 1. DLL3 (SP347) Reactivity in Small Cell Lung Cancer Samples of the Validation Cohort (N = 1362)

Reactivity	Cases	
	No.	%
Not reactive	322	23.6
Reactive	1040	76.4

Abbreviation: DLL3, delta-like protein 3.

any types of cells focally/diffusely in the negative reagent control and stroma/nonneoplastic cells focally/diffusely in the DLL3 (SP347) assay. Background staining greater than 0.5 was considered obtrusive to interpretation of specific staining, and those samples were excluded from analysis. The percentage tumor cell staining was assessed in the DLL3 (SP347)–stained slides. In addition, overall intensity of tumor cell staining on a scale of 0 to 3 in increments of 1 was also assessed in the DLL3 (SP347)–stained slides. Strong cytoplasmic and/or membranous staining of tumor cells was scored as 3, moderate staining as 2, and weak staining as 1. Absence of staining was given a score of 0. Reactivity was defined as samples that had more than 0% DLL3 staining. In addition, the SCLC samples were assessed to identify different staining characteristics.

Discovery Cohort.—Nonspecific background staining was captured and scored for all tested tissue arrays using a scale of 0 to 3 in increments of 0.5. Background staining greater than 0.5 was considered obtrusive to interpretation of specific staining, and those samples were deemed unacceptable. Reactive cores were defined as those tissue cores that stained positive (DLL3 stain intensity or percentage DLL3 cell staining >0).

Neoplastic tissue arrays were scored for specific percentage tumor cell staining. In addition, the overall intensity of tumor cell staining was captured on a scale of 0 to 3 in increments of 1 (intensity scores were given in the same fashion as the scores given for the prevalence study as described above).

Normal, nonneoplastic tissue arrays were scored for DLL3 stain intensity captured on a scale of 0 to 3 in increments of 1. Strong cytoplasmic and/or membranous staining of tissue cores was scored as 3, moderate staining as 2, and weak staining as 1. Absence of staining was given a score of 0.

RESULTS

Validation Cohort

In the validation cohort of 1503 SCLC cases, 141 samples (9.4%) were deemed not evaluable or not acceptable (eg, no tissue/tumor present, crush, necrosis, or high background), and these samples were excluded from the analysis of DLL3 staining and reactivity. Of the 1362 evaluable samples, reactivity was observed in 1040 (76.4%) (Table 1). In addition, the samples exhibited a wide range of staining intensities (Figure 1) and percentage tumor cell staining (bimodal distribution of percentage staining, with 80% of the cases either falling below 25% or above 75% tumor cell staining) (Figure 2).

Staining Characteristics

Upon examination of the SCLC specimens, the following staining characteristics were identified: partial and complete membrane/cytoplasmic staining, granular staining pattern, and homogeneous or heterogeneous staining pattern (Figure 3, A through L).

Discovery Cohort

In the discovery TMAs, 29 samples were deemed not evaluable (eg, no tissue/tumor present, artifacts, or edge artifacts), and these cases were not used in the analysis of reactivity. Samples exhibited a range of staining intensity from weak or moderate to strong in both normal and neoplastic tissues and a range of percentage tumor cell staining in neoplastic tissues.

Reactivity was observed in 24 of 156 evaluable cores (15.8%) in the nonneoplastic TMAs (Table 2). The reactive nonneoplastic cores were composed primarily of normal tissues with a neural or neuroendocrine component. Reactivity was observed in 16 of 65 evaluable cores (24.6%) (1 core was normal tissue adjacent to tumor) in the carcinoma TMA (Table 3) and 80 of 143 evaluable cores (55.9%) in the SCLC TMA.

Table 2. Staining Intensity of Reactive Cores in the Nonneoplastic Tissue Microarrays

Organ	DLL3 Staining	
	Intensity	Localization
Cerebrum	2	Cytoplasm of neurons
Adrenal gland	1	Cytoplasm of adrenal cortical zona reticularis cells
Adrenal gland	1	Cytoplasm of adrenal cortical zona reticularis cells
Pancreas	1	Cytoplasm of pancreatic acinar epithelium and islet cells
Pancreas	1	Cytoplasm of pancreatic acinar epithelium and islet cells
Pancreas	1	Cytoplasm of pancreatic acinar epithelium and islet cells
Hypophysis	2	Cytoplasm of neuroendocrine cells of anterior pituitary
Hypophysis	1	Cytoplasm of neuroendocrine cells of anterior pituitary
Hypophysis	1	Cytoplasm of neuroendocrine cells of anterior pituitary
Testis	2	Cytoplasm of immature seminiferous precursors and Leydig cells
Thyroid gland	1	Cytoplasm of thyroid follicular epithelium cells
Stomach	1	Cytoplasm of chief cells
Liver	1	Cytoplasm of hepatocytes
Liver	2	Cytoplasm of hepatocytes
Larynx	1	Nucleus of mucus glandular epithelium cells
Larynx	1	Nucleus of mucus glandular epithelium cells
Adrenal gland	2	Cytoplasm of adrenal cortical zona reticularis cells
Adrenal gland	2	Cytoplasm of adrenal cortical zona reticularis cells
Adrenal gland	1	Cytoplasm of adrenal cortical zona reticularis cells
Hypophysis	2	Cytoplasm of neuroendocrine cells of anterior pituitary
Hypophysis	2	Cytoplasm of neuroendocrine cells of anterior pituitary
Hypophysis	2	Cytoplasm of neuroendocrine cells of anterior pituitary
Liver	2	Cytoplasm of hepatocytes
Liver	1	Cytoplasm of hepatocytes

Abbreviation: DLL3, delta-like protein 3.

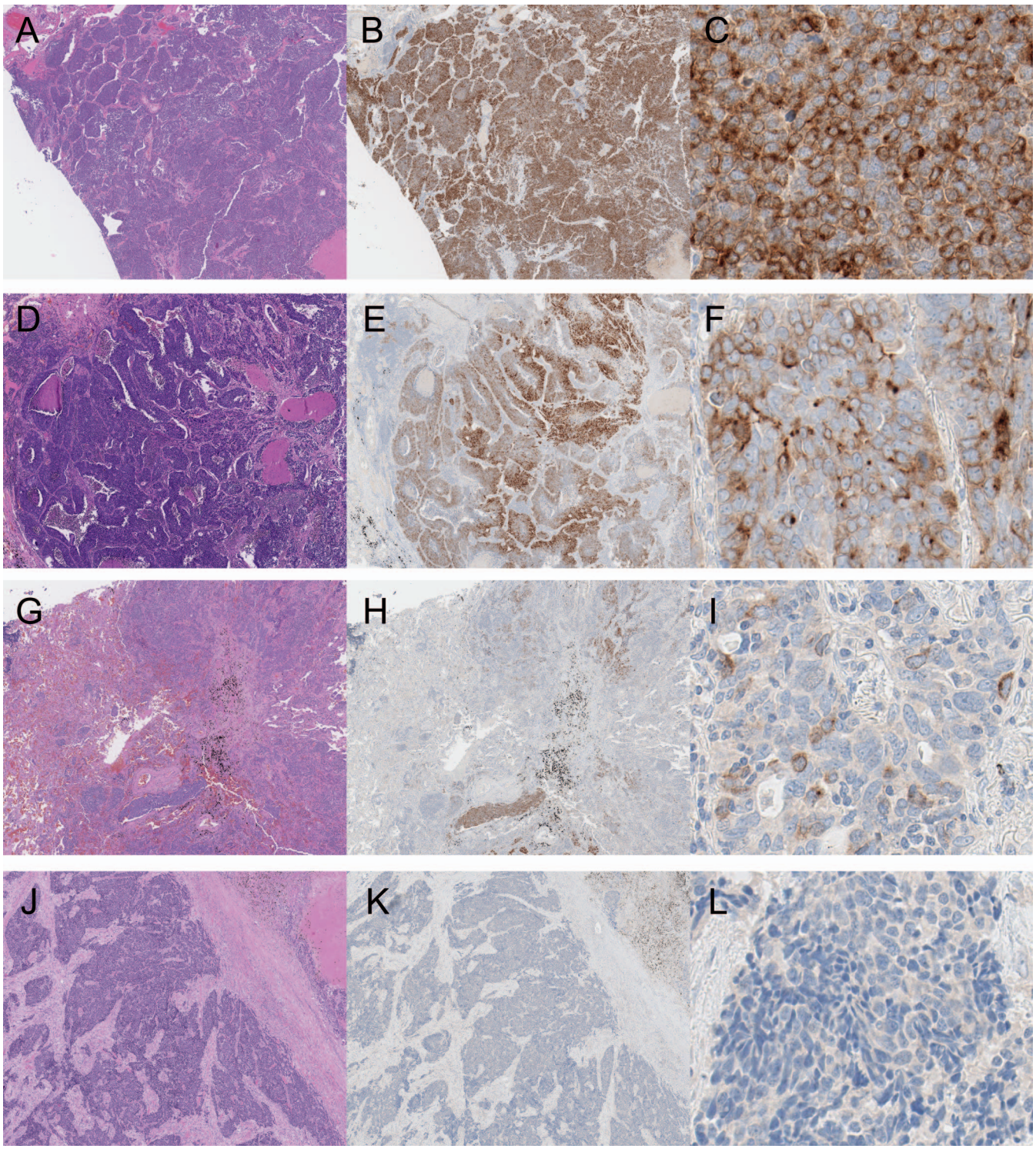

Figure 3. *Representative images showing variable percentages and intensities of delta-like protein 3 (DLL3) (SP347) assay immunohistochemistry (IHC) staining in small cell lung cancer. A through C, High percentage of tumor cell staining with high intensity. D through F, Mid percentage of tumor cell staining with moderate to high intensity. G through I, Low percentage of tumor cell staining with low to moderate intensity. J through L, No staining (hematoxylin-eosin, original magnification ×2 [A, D, G, and J]; DLL3 [SP384] IHC, original magnifications ×2 [B, E, H, and K] and ×40 [C, F, I, and L]).*

DISCUSSION

This is the first study in the literature, to our knowledge, to use the VENTANA DLL3 (SP347) Assay to examine the prevalence of DLL3 protein expression within a cohort of SCLC. In the validation cohort of cases, a high percentage (76.4%) had reactivity to our antibody, exemplified by observable staining with the OptiView detection kit. In addition, the validation cohort of cases showed a bimodal

Table 3. VENTANA DLL3 (SP347) Assay Staining Intensity and Percentage Tumor Cells Staining of Cores in Neoplastic Tissue Microarrays

Organ	Pathology	DLL3 Staining Intensity	% DLL3-Positive Tumor Cells
Cerebrum	Glioblastoma	1	5
Cerebrum	Malignant ependymoma	1	40
Cerebrum	Malignant oligodendroglioma	2	70
Pancreas	Islet cell tumor	1	100
Testis	Seminoma	2	20
Testis	Embryonal carcinoma	1	50
Thyroid	Medullary carcinoma	1	50
Lung	Small cell undifferentiated carcinoma	2	100
Small intestine	Malignant interstitialoma	1	100
Rectum	Moderate malignant interstitialoma	1	100
Liver	Hepatocellular carcinoma	1	20
Cervix	Squamous cell carcinoma	1	2 (staining in immune cells)
Rectum	Malignant melanoma	2	100
Lymph node	Diffuse B-cell lymphoma of right thigh	1	3
Cardiac pericardium	Normal tissue adjacent to tumor	1	NA (cytoplasmic staining on lining cells)

Abbreviations: DLL3, delta-like protein 3; NA, not applicable.

distribution of percentage of cells staining and a wide range of staining intensities.

Three studies of DLL3 expression–level data[8,10,11] showed similar prevalence results. However, all 3 of these studies used a mouse monoclonal antibody, in contrast to the SP347 rabbit monoclonal antibody, and their analysis of positivity had different definitions. In the first study describing DLL3 prevalence in SCLC, the authors used an H-score of 100 or higher as a positive cutoff and found that 120 (72%) of 167 treatment-naïve and 17 (85%) of 20 recurrent and treatment-refractory SCLC cases were positive for DLL3 expression.[8] In the second and third studies, positivity was defined as 1% or more of tumor cells staining. The second study showed that of 48 patients with SCLC or large cell neuroendocrine carcinoma, 42 (88%) had positive staining.[10] Lastly, in a Japanese cohort of 63 patients with SCLC, 52 (83%) were deemed positive for DLL3 expression.[11] In summary, DLL3 (SP384) prevalence data are consistent with the data in the literature using other antibodies, showing that there are high rates of DLL3 protein expression in SCLC.

Additionally, reactivity was observed in a small percentage of nonneoplastic and neoplastic tissues, and was significantly higher within discovery SCLC TMAs in our study. This is consistent with a study by Saunders et al[8] that showed low prevalence of DLL3 in normal, nonneoplastic tissues. Also, this study demonstrated that the reactive tissues were primarily of neural or neuroendocrine origin, which corresponds with the known characteristics of DLL3 in mRNA studies.[8,12]

Herein, we present data that show the prevalence of DLL3 expression in a cohort of 1503 cases and also show that the VENTANA DLL3 (SP347) Assay is reactive with recognizable staining patterns that can be used for determining the level of DLL3 expression in SCLC.

References

1. American Cancer Society. Cancer facts & figures 2017. https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2017/cancer-facts-and-figures-2017.pdf. Accessed March 26, 2018.

2. PDQ Adult Treatment Editorial Board. Small cell lung cancer treatment (PDQ®): health professional version. Bethesda MD: National Cancer Institute. http://www.ncbi.nlm.nih.gov/books/NBK65909/#CDR0000062945__1. Published January 20, 2017. Accessed March 26, 2018

3. Joshi M, Ayoola A, Belani CP. Small-cell lung cancer: an update on targeted therapies. *Adv Exp Med Biol*. 2013;779:385–404.

4. William WN Jr, Glisson BS. Novel strategies for the treatment of small-cell lung carcinoma. *Nat Rev Clin Oncol*. 2011;8(10):611–619.

5. Von Pawel J, Jotte R, Spigel DR, et al. Randomized phase III trial of amrubicin versus topotecan as second-line treatment for patients with small-cell lung cancer. *J Clin Oncol*. 2014;32(35):4012–4019.

6. National Comprehensive Cancer Network. NCCN guidelines for treatment of cancer by site: small cell lung cancer. https://www.nccn.org/professionals/physician_gls/default.aspx. Accessed September 6, 2018.

7. Crabtree J, Singleton C, Miele L. Notch signaling in neuroendocrine tumors. *Front Oncol*. 2016;6:94.

8. Saunders LR, Bankovich AJ, Anderson WC, et al. A DLL3-targeted antibody-drug conjugate eradicates high-grade pulmonary neuroendocrine tumor-initiating cells in vivo. *Sci Transl Med*. 2015;7(302):302ra136.

9. Jiang T, Collins BJ, Jin N, et al. Achaete-scute complex homologue 1 regulates tumor-initiating capacity in human small cell lung cancer. *Cancer Res*. 2009;69(3):845–854.

10. Rudin CM, Pietanza MC, Bauer TM, et al. Rovalpituzumab tesirine, a DLL3-targeted antibody-drug conjugate, in recurrent small-cell lung cancer: a first-in-human, first-in-class, open-label, phase 1 study. *Lancet Oncol*. 2017;18(1):42–51.

11. Tanaka K, Isse K, Fujihira T, et al. Prevalence of Delta-like protein 3 expression in patients with small cell lung cancer. *Lung Cancer*. 2018;115:116–120.

12. DLL3. The Human Protein Atlas website. https://www.proteinatlas.org/ENSG00000090932-DLL3/tissue. Accessed March 26, 2018.